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Multiplexed heat shock protein microarray as a screening platform for the selection of novel drug compounds

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Abstract

In diseases such as cancer, Alzheimer's disease or malaria, disease-related proteins take advantage of the heat shock protein (HSP) control system for their own activation or maturation. There is a quest to find inhibitors that specifically bind to the HSPs. Here, we report on a novel multiplexed assay system for inhibitor screening based on a protein microarray (MA) technique that was developed for routine applications with storable MAs. Purified HSPs are printed as full-length proteins on microarrays and used as a drug target for the screening of new inhibitors. Derivatives obtained by a combination of biological and chemical synthesis were tested as competitors of ATP with a suggested affinity for several HSP proteins which are hHSP from human, AtHSP83 (*Arabidopsis thaliana*) and HtpG from *Helicobacter pylori*. Some of these new derivatives exerted selectivity between human and bacterial heat shock proteins. Printed human HSP90 was used to test the binding of denatured proteins on the client binding site of human HSP90, since the full-length HSP maintains the capability to bind putative clients or cochaperones. Initial data revealed that the microarray application can be used to identify directly elevated heat-shock protein levels in cancer cell lysates. We suggest that microarray-based assaying of HSP levels can be used as a marker for determining stress levels.

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Abbreviations: 17-AAG, 17-N-allylamino-17-demethoxygeldanamycin; BSA, bovine serum albumin; Cy3, carbocyanin 3; DMSO, dimethylsulfoxid; HEPES, 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid; HSP, heat shock protein; HSP83, heat shock protein at molecular weight 83 kilodalton; At, Arabidopsis thaliana; HtpG, high temperature protein G; IC50, half minimal inhibitory concentration

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Background

Heat shock proteins (HSPs) are molecular chaperones, which help other proteins to mature and fold to their native structure even under extreme environmental conditions. HSPs are essential for the maintenance and function of the proteome [1-4]. Due to their special key position mediating a rescue mechanism, HSPs are targets for drug development in diseases such as cancer [5-8]. Inhibitors or drugs lower increased cellular HSP90 activity and thereby increase misfolded client proteins with oncogenic potential that arise as a result from mutations [9-12]. This ideally leads to the death of the cell or in special cases to the reduction of a pathogen, when a suitable HSP inhibitor is present.

Within this study we used a recently reported HSP microarray for the target-oriented screening of HSP inhibitors [13]. While in our previous publication [13] the microarray was solely used to screen molecules for their potential to inhibit the ATP binding sites of HSP90 and HtpG, this short communication aims to further broaden the range of applications:

(i) Since the ATP - binding sites exist in all HSP target proteins, different target proteins can be examined in parallel in a multiplexing approach. The assay is performed with tiny amounts per spot (800-1600 picoliters) without limiting the sensitivity. The recombinant purified target proteins bind labeled ATP; binding and displacement by potential inhibitors can be detected optically in the microarray format. In this communication, we demonstrate the multiplexing capability by using human HSP90, bacterial HtpG, and plant-derived HSP83.

(ii) The assay can also be performed with HSP originating from cell lysates by directly printing the lysates to the microarray. We show that this lysate microarray can be used to detect elevated HSP titers and hypothesise potential diagnostic applications.

(iii) Furthermore, HSP interactions with labeled proteins/peptides can be detected, resulting in the possibility to screen the client binding site of HSP as a further potential target site for drug development.

Results and Discussion

Purified HSPs (human HSP90α, plant AtHSP83, *H. pylori* HtpG) have been printed in parallel onto a microarray surface. Fluorescently (Cy3) labeled ATP directly competed with different natural product derived heat shock protein inhibitors. These natural products were accessed by a combination of chemical synthesis and mutational biosynthesis [13-15]. The potential inhibitors were applied in different concentrations ranging from 50 µM to 50 pM and the data collected were used to calculate IC_{50} values as described by Schax et al. [13]. Figure 2a exemplifies the results of the competition assay for purified AtHSP83. It could be demonstrated that an efficient competition between labeled ATP and the used geldanamycin derivative 17 AAG and bromo-reblastin occurs (Fig. 1) as indicated by the calculated IC_{50} values of 291 nM for 17 AAG and 79 nM for bomo-reblastin (Fig. 2a). It was demonstrated that compounds **2** and **3** were also able to effectively displace the labeled ATP from purified HSP83 from *A. thaliana* (Fig. 2a). The results of screening several potential inhibitors against human and bacterial HSPs are summarized for comparison in the heat map shown in Figure 2b. Our results indicate that it is possible to compete labeled ATP with different efficiencies for different HSPs. Using the HSP microarray new compounds that bind specifically to one of the HSPs were identified from a small drug library synthesized and characterized as described recently [13-15].

The HSP microarray has the advantage to allow the multiplexed testing of an `HSP-zoo´ composed of different HSPs derived from different organisms against a large arsenal of potential drugs.

The HSP microarray is a novel miniaturized assay system used for determining inhibition of the ATP-binding pocket in HSPs from human (HSP90α) as well as bacterial sources (HtpG from *H. pylori*) by small molecules and this could potentially allow HSP screening under high throughput conditions. An important outcome of this preliminary study is the observation that drug candidates derived from benzoquinone geldanamycin

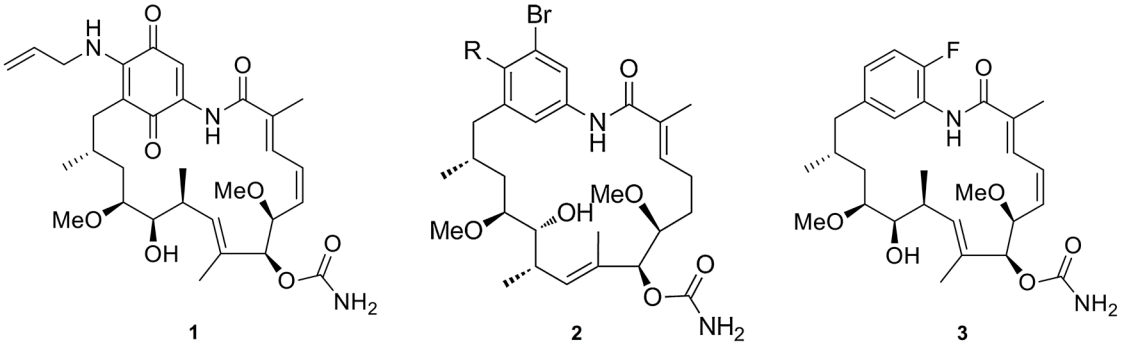

Figure 1. Structures of 17 AAG (1) and the non-quinone geldanamycin bromo-reblastin derivative (2) and 18-dehydrox-19-fluoro-reblastatin (3).

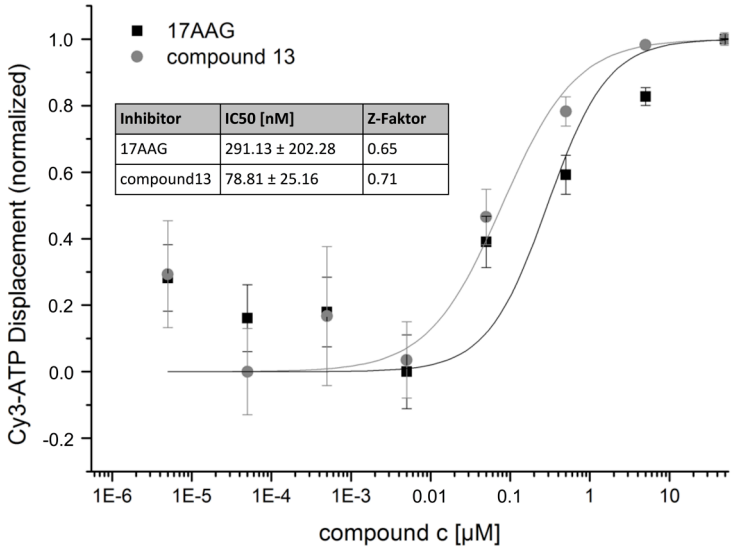

Figure 2a. Typical displacement assay of Cy3-ATP on AtHSP83 by geldanamycin derivative. Purified AtHSP83 was printed on the microarray surface and Cy3-ATP was competed by a geldanamycin derivative, synthesis and properties given in Schax et al., [13]. The IC_{50} value was obtained from dose-response curve fittings.

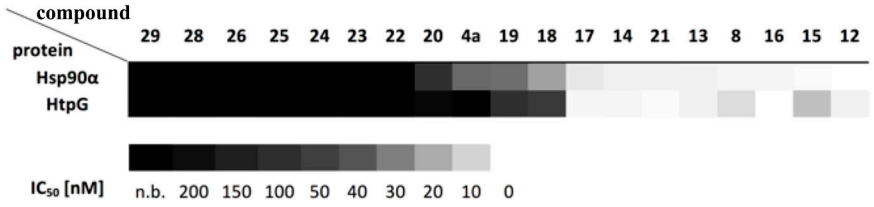

Figure 2b. Heat map of the calculated IC_{50} values of HSP90α from *H. sapiens,* HtpG from *H. pylori* and HSP83 from *A. thaliana* for several tested potential inhibitors in a competitive displacement assay. Numbers as indicated have been described before [13]. Different grey tones from black to white indicate the potency of the inhibitor. White corresponds strong inhibitory effects, whereas black shows no effects for the competition with ATP for the binding pocket.

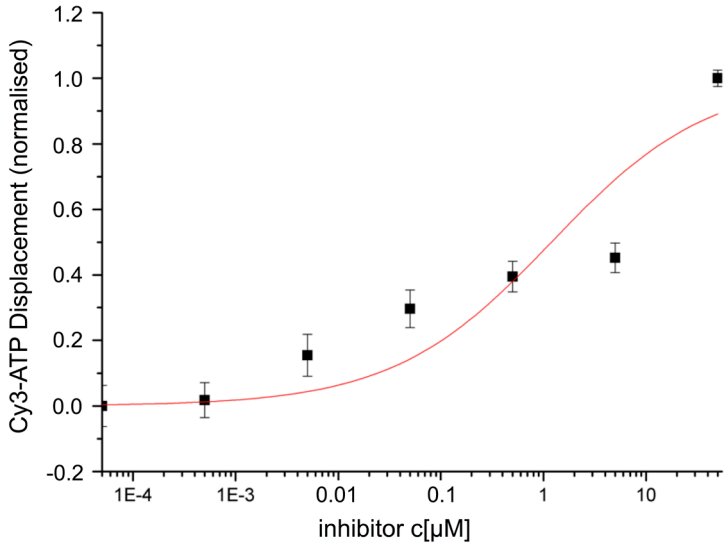

Figure 2c. Competitive assay of Cy3-ATP by geldanamycin derivative 18-dehydrox-19-fluoro-reblastatin [13] on cancer cell lysates. Concentrated human cancer cell lysate obtained from cell culture was printed on microarray surface and bound Cy3-ATP was competed by a geldanamycin derivative. The IC_{50} value was obtained from dose-response curve fittings.

like 17-AAG are selective between human HSP, plant HSPs and bacterial HtpG [13]. This assay will allow us to search for small molecules that selectively inhibit pathogenic bacterial HSPs [13] and not the human HSP90α.

To explore prospective diagnostic applications of the HSP microarray, we have investigated the potential of the microarray to directly estimate the HSP level by printing of cancer cell lysates. Cancer cell lysates were printed on the microarray, and it could be shown that the labeled ATP was competed by a geldanamycin derivative 18-dehydrox-19-fluoro-reblastatin (Fig. 2c). Interestingly, the geldanamycin derivative 18-dehydrox-19-fluoro-reblastatin (Fig. 1), which is fluorinated at the phenyl-ring, is a strong competitor of ATP of all investigated HSPs with different displacement potencies [13]. This demonstrates the possibility to test individual tissues for HSP levels and broadens the applicability of the HSP microarray for the diagnostic field using human HSPα as a cell stress marker and perhaps also in plant tissues. This may also enable the detection of HSP-related cancer cells in liquor or tissues after tumor dissection.

While the above mentioned applications of the HSP microarray exploit the binding of ATP and corresponding inhibitors to the ATP binding site of HSP, HSP offers more sites that could be used as drug binding sites. One of these sites is the client binding site. To investigate whether the full-length human HSP90α printed on the microarray is able to bind client proteins, we have screened the binding of different proteins in native and denatured form (Fig. 3). Luciferase, plant calmodulin and bacterial potassium channel KcsA were tested as potential client proteins; the proteins were purified and labeled with Cy3. Denatured proteins were obtained by heat denaturation and binding of denatured proteins was compared to binding of native proteins. All binding tests were performed under different conditions, only the presence of Tween20 enabled binding, whereas other detergents (octyl glucosid, triton X100, or NP40) or no detergent in the binding buffer did reduce client binding completely.

It could be shown that heat denaturation was not required for KcsA binding. In case of luciferase, only the denatured protein binds to the printed HSP90, while no binding of native luciferase was observed. This indicates the specific binding of the denatured protein to the client-binding site of HSP, which is expected to show increased binding of denatured and thus misfolded proteins. In contrast, denatured or native calmodulin does not bind to HSP90. Since HSP90 detects unfolded proteins only, a higher heat stability of calmodulin may explain the loss of binding. The successful binding of the other proteins to HSP indicates that the client-binding site of printed HSP90 is active and could serve as an additional target-site for screening of drug compounds.

In summary, the described HSP microarray bears the potential to screen against HSP derived from further sources including other pathogens and plants. Target-oriented screening was successfully used to estimate HSP levels in cancer cell lysates to enable diagnostic application, which may serve as a novel stress test. The microarray could thus enable a direct testing of tumor tissues after dissection from patients. The tissue is lysed and printed onto the microarray for determination of an inhibitor profile against the complete the ATP proteome including elevated protein concentrations of Hsp90 in the case of some cancer types. At this point it is a diagnostic tool and indicates whether the tumor has modifications from TP53 and corresponding HSP pathways (50% of all cancer types) with the possibility to give a prognosis and choice and for subsequent therapies

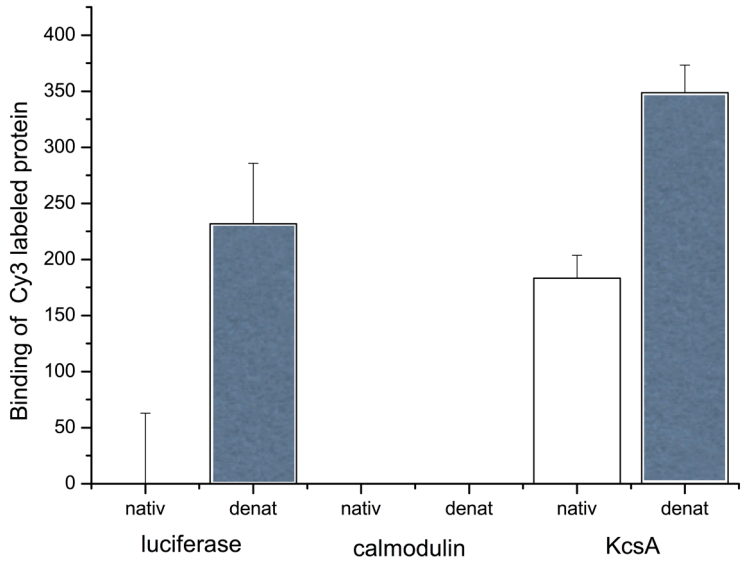

Figure 3. Binding of labeled potential client proteins to HSP90α. Signal intensities were analysed at the wavelength 532 nm with a gain of 200.

(this is under development together with the Medical High School Hannover, MHH; pers. comm.). This is a strategy to develop tools for personalized medicine with applications for other dissected materials or liquors from patients.

In addition to the well-characterized ATP binding site of HSP90, other HSP90 sites can be tested e.g. by binding of client proteins or co-chaperones to improve novel drugs against the corresponding binding sites. As a main advantage, the concept of the assay is general and should be transferable to other ATP-dependent HSPs and enzymes.

Experimental Section

Preparation of geldanamycin derivatives

All geldanamycin derivatives, except for 17-AAG, used in this study were obtained as previously reported [13, 14]. The synthetic strategy relied on mutasynthesis using a mutant strain of *S. hygroscopicus* var. *geldanus* that was blocked in the biosynthesis of the starter unit, 3-amino-5-hydroxybenzoic acid (AHBA) [14]. 17-AAG and radicicol are commercially available (Biomol).

Protein preparations

The cDNA of HSP83 from *A. thaliana* was cloned into a petSUMO plasmid, transformed into the *Escherichia coli* Rosetta strain, overexpressed and purified as previously described [13]. Synthesis and purification of the target proteins HSP90α and HtpG from *Helicobacter pylori* were produced as described before [13] and the putative clients were produced in Escherichia coli. The bacterial potassium channel was produced and purified as described by Barbier et al., [16]. The firefly luciferase was obtained after transformation of the pBest-Luc (Promega) into *Escherichia coli* Bl21DE3 strain and luciferase activity was tested after purification as described by [17] The cDNA of calmodulin was cloned into pTrcHis2TOPO plasmid, transformed into the *Escherichia coli* Bl21DE3 strain. Purified proteins were frozen at a concentration of 1-3 mg ml^{-1} in liquid nitrogen and stored at 80°C.

Direct competitive assay

The HSP microarray assay was performed as described by Schax et al., (2014) using 4 x 5 matrices of proteins printed on UniSart® 3D nitro slide (Sartorius Stedim Biotech, Göttingen, Germany) using a GeSim Nano-Plotter™ (GeSim, Grosserkmannsdorf, Germany) equipped with a nanotip pipette. The printed proteins on the slides were incubated directly with a mixture of 100 nM Cy3-ATP and potential inhibitors. The potential inhibitors were used in different concentrations ranging from 50 µM to 50 pM. The competitive assay was carried out in binding buffer (20 mM HEPES-KOH, pH 7.3, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Na_2MoO_4, 0.01% v/v Tween 20, 2 % v/v DMSO, 0.1 mg ml^{-1} BSA) for 16 h at 4°C. Detection of the bound labeled ATP was performed using a GenePix 4000B Laser Scanner (Molecular Devices, Inc., Sunnyvale, CA, USA) with 532 nm emission wavelength, laser power 33 %, PMT gain 350. Quantification was performed using GenePix Pro 6.1 (Molecular Devices, Inc., Sunnyvale, CA, USA) and ImaGene 5 of BioDiscovery, Inc. (Hawthorne, CA, USA). The dose-response curves were calculated with Origin 7G (OriginLab Corporation, Northampton, MA, USA) and fitted with the non-linear function logistic, A1=0, A2=1 to obtain IC_{50} values.

Cell lysate preparation

The frozen cell pellet consisting of 10^5 A549 cells were thawed on ice and resuspended in 100 µl phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Afterwards they were mixed with 1 ml of Tris-buffered saline (50 mM Tris-Cl, pH 7.6, 150 mM NaCl). The lysis of the cells was performed on ice with ultrasonic energy (pulse duration 0.6 sec, 5 repeats à 30 sec, and break in-between of 60 sec). The cell debris were removed by centrifugation at 14,000 xg, 4°C and for 30 min. Using centrifugal concentrators with a polyethersulfon membrane, pore size 0.2 µm (Sartorius Stedim Biotech, Göttingen, Germany), the supernatant was filtered for 3 min at 4°C at 14,000 xg. The collected flow-though was concentrated with the help of an ultrafiltration unit (polyethersulfon membrane) with a molecular weight cut-off of 10 kDa (Sartorius Stedim Biotech, Göttingen, Germany) at 14,000 xg for 20 min at 4°C. Furthermore, the buffer was exchanged to storage buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 10% v/v Glycerin, 6 mM 2-mercaptoethanol) and the concentration of protein in the final solution was 2.52 mg ml^{-1}.

Protein labeling and binding to HSP90α

Purified Luciferase, calmodulin and KcsA were labeled with Cy3 (Cy3 Mono-Reactive Dye Pack, Amersham, Piscataway, NJ) at a protein concentration of 1 mg ml^{-1} as described by Walter et al. [18] and unbound label was removed through centrifugal concentrators with a cut-off of 10 kDa. The resulting Cy3/protein-ratios were 0.04 for calmodulin, 0.33 for KcsA, and 1.65 for luciferase. Before incubation with printed HSP90α one fraction of the labeled proteins were denatured at 45°C for 10 min. Prior incubation with printed HSP90 the protein concentration of each potential client protein was adjusted to 2 ng $µl^{-1}$ in 20 mM Hepes, 50 mM KCl, 5 mM $MgCl_2$, 30 mM Na_2MoO_4, 1mM DTT, 0.01 % Tween20, 1 mg ml^{-1} BSA, pH 7.3. For each client protein, native

and denatured forms of labeled protein were incubated onto the microarray over night at 4°C. The microarrays washed with 20 mM Hepes, 50 mM KCl, 5 mM $MgCl_2$, 30 mM Na_2MoO_4, 1mM DTT, 0.01 % Tween20, 1 mg ml^{-1} BSA, pH 7.3 for 10 min three times. The evaluation of the binding and treatment of the slides was performed as described before [13].

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